

A *Drosophila* CREB/CREM Homolog Encodes Multiple Isoforms, Including a Cyclic AMP-Dependent Protein Kinase-Responsive Transcriptional Activator and Antagonist

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We have characterized a *Drosophila* gene that is a highly conserved homolog of the mammalian cyclic AMP (cAMP)-responsive transcription factors CREB and CREM. Uniquely among *Drosophila* genes characterized to date, it codes for a cAMP-responsive transcriptional activator. An alternatively spliced product of the same gene is a specific antagonist of cAMP-inducible transcription. Analysis of the splicing pattern of the gene suggests that the gene may be the predecessor of the mammalian CREB and CREM genes.

Activation of the cyclic AMP (cAMP) signal transduction pathway can have long-lasting global consequences through its influence on the expression of specific genes. In mammalian systems, many of the known cAMP-responsive genes serve important neural and endocrine roles (38, 54, 63, 70).

Some members of the CREB family of transcription factors in mammals are known to participate in the control of gene expression by cAMP (13, 25, 32, 36). Proteins of this family consist of two major domains. The carboxyl-terminal portion contains a basic region-leucine zipper (bZIP) domain, involved in sequence-specific DNA binding and protein dimerization (9, 33, 40, 74, 79). The remainder of the protein comprises the activation domain. This contains regions that can presumably interact with other components of the transcription machinery and with signal transduction pathways to influence gene expression (10, 25, 27, 43). CREB proteins can bind as dimers to a conserved palindromic DNA sequence, the cAMP-responsive element (CRE; consensus sequence, 5' TGACGTCA 3'). CRE sequences have been characterized from upstream control regions of cAMP-responsive genes (15, 39, 55, 63, 80) and have been identified as the *cis*-acting component of the CREB-mediated transcriptional response to cAMP.

cAMP-responsive transcriptional activation from CREs appears to require the presence of cAMP-dependent protein kinase (PKA) in the nucleus (50). Persistent elevation of cAMP levels can lead to the migration of free catalytic subunits of PKA to the nucleus (5, 22, 32, 34, 56). Experimentally, elevation of PKA levels or injection of PKA into the nucleus can substitute for all earlier cytoplasmic steps in this signal transduction pathway (7, 32, 51, 60, 61). In the nucleus, PKA is believed to phosphorylate and activate nuclear substrates that include a subset of CREB family proteins (3, 50). Phosphorylation of a particular serine residue by PKA is obligatory for cAMP-dependent transcriptional activation by the best characterized of the cAMP-responsive activator proteins, CREB (28, 43).

Only two other genes in the CREB family, besides CREB itself, are known to code for PKA-responsive activators: CREM (23, 25) and ATF-1 (46, 59). Other CREB family mem-

bers that have been tested do not appear to respond to PKA (47, 67). The CREM gene, besides producing PKA-responsive transcriptional activators, gives rise to isoforms that explicitly antagonize cAMP-dependent transcription (23, 24).

We undertook the cloning of *Drosophila* CREB/ATF family members with the specific aim of obtaining tools for examining the involvement of cAMP-dependent gene expression in *Drosophila* long-term memory. We have isolated and characterized a *Drosophila* CREB family gene that produces a number of alternatively spliced isoforms. This gene, which we call *dCREB2*, is the same gene previously reported as *dCREB-B* and partially characterized by Usui et al. (73) on the basis of one cloned cDNA isoform. That isoform (corresponding to the *dCREB2-c* isoform illustrated in Fig. 5) did not encode a PKA-responsive transcriptional activator. Among the isoforms that we have characterized is one that is a PKA-responsive transcriptional activator (*dCREB2-a*) and another that is an antagonist of PKA-responsive transcriptional activation (*dCREB2-b*). In our communications, we have used the nomenclature *dCREB2-x* to differentiate the various alternatively spliced forms, where *x* is a letter code signifying the particular isoform (see Fig. 5). Furthermore, our analysis of the genomic organization of *dCREB2* identified additional coding exons, requiring a numbering scheme different from that in reference 73. For these reasons, in this report we will use our own nomenclature for the gene.

dCREB2 appears to be closely related to, and perhaps an ancestral form of, both of the mammalian genes CREB and CREM. The presence of a PKA-responsive transcriptional activator from the CREB family in *Drosophila melanogaster* suggests that this organism may share mechanisms for the control cAMP-dependent gene expression with the CREB-mediated system of mammals. The characterization of the various gene products from this gene will provide useful biological tools for examining the involvement of cAMP-dependent gene expression in long-term memory (76, 77) and in other areas of *Drosophila* biology where cAMP is an important second messenger.

MATERIALS AND METHODS

Expression cloning of *dCREB2*. Standard protocols for expression cloning by DNA-binding activity (4, 66) were followed except as noted. A double-stranded 3xCRE oligonucleotide based on an adenovirus E4 CRE (45) was synthesized

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LANE	1	2	3	4	5	6	7	8	9	10	11	12
PROBE	WT	M	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
COMPETITOR	-	-	S	S	NS1	NS1	NS2	NS2	NS3	NS3	NS4	NS4
MOLAR EXCESS	-	-	20	100	20	100	20	100	20	100	20	100

COMPLEX

FREE PROBE

FIG. 1. Gel mobility shift assays of bacterially expressed dCREB2. All reactions contained labeled wild-type (WT) 3xCRE DNA probes except the reaction in lane 2, which contained labeled mutant (M) 3xCRE probe. Competitors were added at two different molar ratios. Reactions in lanes 3 and 4 contained specific (S: 3xCRE) competitor oligonucleotides, while the reactions in lanes 5 to 12 contained nonspecific competitors (NS1 to NS4).

absence of exon 4. They also had differences in their 5' and 3' untranslated sequences.

Chromosomal in situ hybridization using a *dCREB2* probe resulted in diffuse labeling centered at 17A2 on the X chromosome, in agreement with the results of Usui et al. (73). This region contains several lethal complementation groups (18).

DNA-binding properties of *dCREB2-b*. The DNA-binding activity of dCREB2-b was tested in a gel mobility shift assay (Fig. 1). Bacterial extracts expressing the dCREB2-b protein retarded the migration of a 3xCRE probe. The protein had lower but detectable affinity for a mutated 3xCRE oligonucleotide (compare lanes 1 and 2). Competition experiments using unlabeled competitor oligonucleotides showed that the binding of dCREB2-b to 3xCRE was specific (lanes 3 to 12), with higher affinity for CRE sites than to nonspecific DNA. Together with the conserved amino acid sequence, this functional similarity suggested that *dCREB2* was a CREB family member.

Expression pattern of *dCREB2*. Northern blot analysis of poly(A)⁺ RNA from larval stages and heads and bodies of adult flies (Fig. 2A) showed a complex pattern of bands, with at least 12 different-size transcripts apparent. Two bands of approximately 0.8 and 3.5 kb were common to all of the stages. The adult head contained transcripts of at least six sizes (0.8, 1.2, 1.6, 1.9, 2.3, and 3.5 kb). In situ hybridization to RNA in *Drosophila* head tissue sections, using a riboprobe with antisense sequences for the dCREB2 bZIP region, resulted in signal in most or all cells. In the brain, cell bodies, but not neuropil, were stained (Fig. 2B).

***dCREB2* has alternatively spliced forms.** Our initial transfection experiments showed that the dCREB2-c isoform was not a PKA-responsive transcriptional activator (data not shown), a result confirming preliminary observations (73). The complex developmental expression pattern of transcripts from *dCREB2*, plus information from the mammalian *CREM* gene that alternative splicing was required to generate PKA-responsive activators (23, 25, 42), suggested that additional domains might be required for activating isoforms from *dCREB2*.

RT-PCR was used to identify new coding exons. Comparison of *dCREB2* genomic DNA sequence with that of cDNAs indicated the general exon/intron organization and assisted in the search for additional exons. Sense and antisense primers spaced across the *dCREB2-b* cDNA were synthesized and used pairwise in RT-PCRs primed with *Drosophila* head mRNA to look for these new coding regions. Positions of the PCR primers used in this analysis are shown in Fig. 3. Reactions with

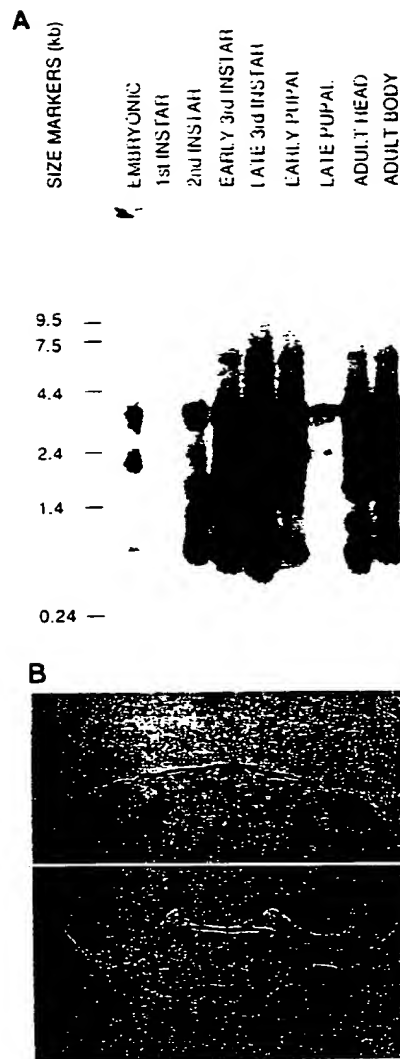


FIG. 2. (A) Northern blot analysis of *dCREB2* transcripts. Poly(A)⁺ RNA samples from different developmental stages were separated and probed with an antisense *dCREB2* probe. (B) *dCREB2* RNA in a representative (medial) tissue section from *Drosophila* head. An antisense riboprobe to *dCREB2* was hybridized to serial frontal sections of wild-type (Can-5) flies. Sections hybridized with the corresponding sense riboprobe showed no signal.

primer pair A and B, hybridizing in exons 5 and 7, generated two products, one of the size predicted by our cDNA clones and one that was larger. Cloning and sequencing of the larger product suggested the presence of exon 6. A primer within exon 6 (primer C) was synthesized, end labeled, and used to screen a *Drosophila* head cDNA library. Two clones were isolated, sequenced, and found to be identical. This splicing isoform, *dCREB2-d*, confirmed the splice junctions and exon sequence inferred from the RT-PCR products.

Initial attempts to isolate exon 2 proved difficult. We conceptually translated the genomic sequence that separated exons 1 and 3 in all frames and identified one relatively long ORF. Of three antisense primers (D, E, and F) synthesized on the basis of the genomic sequence, only one (primer D) fell within the ORF. Three sets of RT-PCR each using a different one of the three antisense primers paired with primer G, a

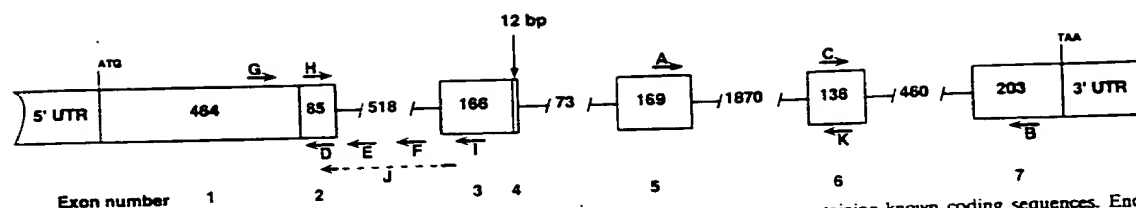


FIG. 3. Schematic *dCREB2* genomic region showing PCR primer locations. Boxes represent exons containing known coding sequences. Enclosed numbers indicate lengths of coding regions in base pairs. Lines between boxes indicate introns and approximate lengths in base pairs. Horizontal arrows represent PCR primers discussed in the text. UTR, untranslated region.

sense primer in exon 1, were run in parallel. Only the reaction that used primer D produced a PCR product. The sequence of this product matched a continuous stretch of nucleotides from the genomic sequence, extending 3' from exon 1 past the splice junction in the *dCREB2-b* cDNA to the location of primer D. This fragment suggested that exon 1 might be extended in some mRNAs by use of an alternative 5' splice site located 3' to the site used to make *dCREB2-b*. On the basis of the newly identified exon sequences, we made primer H, which when used in a PCR with primer I in exon 3 generated a new product whose sequence established the location of the alternative 5' splice site. The sequence added to exon 1 by alternative 5' splice site selection is designated exon 2. The exon 2 sequence also showed that the same 3' splice site was used both in the cDNAs that we originally isolated and in those used for the RT-PCR product. To independently verify this alternative splicing pattern, we carried out RT-PCR using primer J, which spans the 3' splice junction of exon 2, in combination with primer G in exon 1. The sequence of the product corroborated the splice junctions of exon 2 shown in Fig. 3.

To determine if exons 2 and 6 could be coordinately spliced into the same molecule, we carried out an RT-PCR with primers H and K, located in exons 2 and 6, respectively. The reaction produced a product of the size predicted for a fragment containing exons 2, 3, 4, 5, and 6, and the identity of this product was confirmed by extensive restriction analysis.

dCREB2 is a *Drosophila* CREB gene. Figure 4 shows the DNA sequence and inferred amino acid sequence of *dCREB2-a*, the ORF which results from combining all of the identified *dCREB2* exons. The translation start site indicated for *dCREB2-a* is likely to be authentic because (i) stop codons occur upstream from this ATG in all reading frames in our *dCREB2* cDNAs (sequences not shown), (ii) this ATG was selected by computerized analysis (68) as the best ribosome binding site in the DNA sequence that contains the ORF, and (iii) use of the next ATG in the ORF (480 nucleotides downstream) would not predict a protein that would be a PKA-dependent activator (see below). This does not exclude the possibility that internal translation initiation sites are used in this transcript, as happens with the CREM gene's S-CREM isoform (14).

The *dCREB2-a* ORF predicts a protein of 360 amino acids. A computerized amino acid sequence homology search (71) with the predicted *dCREB2-a* protein sequence identifies CREB, CREM, and ATF-1 gene products as the closest matches to *dCREB2-a*. As noted previously (73), amino acid homology is particularly striking between *dCREB2* and these three mammalian CREBs in the carboxyl-terminal bZIP domain (Fig. 4B). Amino acid homology is much less strong, but still present, in the activation domain. The predicted *dCREB2-a* product has a region of amino acids containing consensus phosphorylation sites (58) for PKA, calcium/calmodulin-dependent kinase II, and protein kinase C, analogous to the more extensive P-box or kinase-inducible domains defined

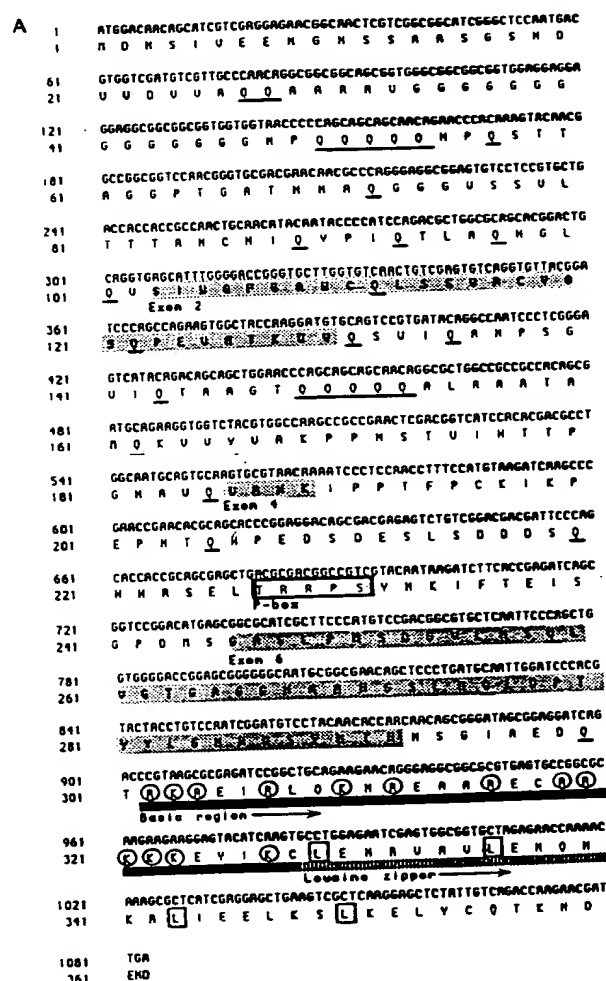


FIG. 4. (A) DNA sequence and predicted amino acid sequence of the *dCREB2-a* coding region. The basic region and leucine zipper domains are indicated by solid and broken bold underlining, respectively. Positively charged residues in the basic region are circled; periodic leucines in the zipper motif are boxed; glutamines in the activation domain are underlined. The short amino acid motif with target sites for kinases, starting at residue 227, is indicated by a bold outline. Sequences specified by alternatively spliced exons 2, 4, and 6 are shaded. (B) Amino acid sequence comparison of bZIP domains of *dCREB2*, mammalian CREB, CREM, and ATF-1. Differences between *dCREB2* and CREB are boxed.

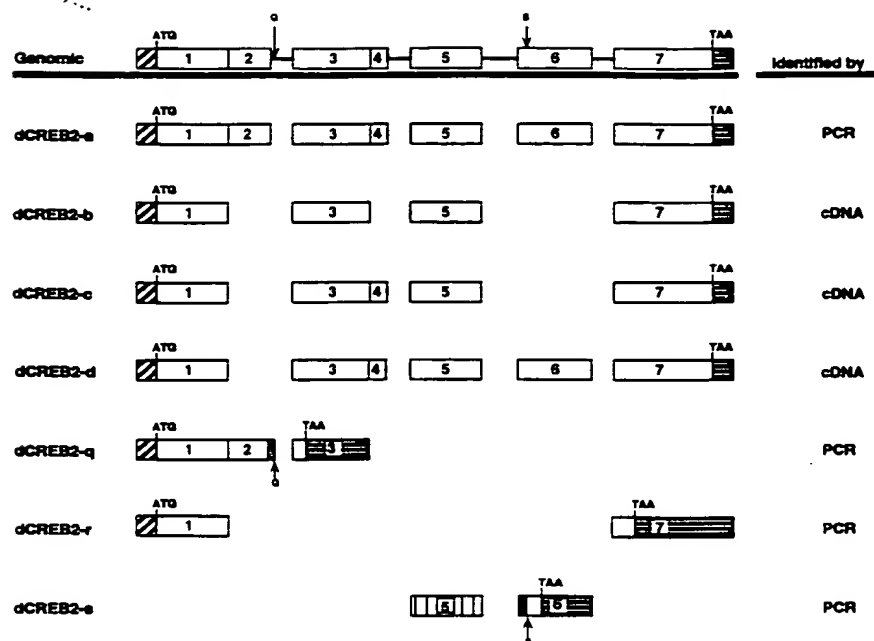


FIG. 5. Diagram of *dCREB2* isoforms. Exon boundaries are defined with respect to *dCREB2-a*. Diagram is not drawn to scale.

in CREB, CREM, and ATF-1. Like the P boxes in CREB and CREM- τ , the *dCREB2-a* P box is located carboxyl terminal to a glutamine-rich region. As in CREM τ , the *dCREB2-a* P box lies between a pair of alternatively spliced domains implicated in transcriptional activation (42).

Figure 5 shows the exons present in each of the *dCREB2* alternative splice forms that we have detected, both as cDNAs and by RT-PCR. The splice products of *dCREB2* fall into two broad categories. One class of transcripts (*dCREB2-a*, *-b*, *-c*, and *-d*) employs alternative splicing of exons 2, 4, and 6 to produce isoforms whose predicted protein products all have the bZIP domains attached to different versions of the activation domain. Members of the second class of transcripts (*dCREB2-q*, *-r*, and *-s*) all use alternative splice site selection. Splice forms q and s are generated from alternative 5' and 3' splice sites respectively, while form r is a direct splice from exon 1 to exon 7. These splicing variations change the reading frame and result in translation termination at various positions 5' of the bZIP domain. Thus, they predict a set of truncated activation domains lacking the DNA-binding or dimerization activity that the basic region and leucine zipper provide.

Two different *dCREB2* isoforms, *dCREB2-a* and *dCREB2-b*, have opposite roles in PKA-responsive transcription. The ability of *dCREB2* isoforms to mediate PKA-responsive transcription was tested in F9 cells. These cells have been used extensively to study CREB-dependent activation because their endogenous cAMP-responsive transcription system is inactive (28, 49). In this system, expression constructs for candidate cAMP-responsive transcription factors are transiently transfected with and without a construct expressing the PKA catalytic subunit. CREB-dependent changes in gene expression are measured by using a cotransfected reporter construct that has a CRE-containing promoter fused to coding sequences of the bacterial CAT gene.

In this assay, *dCREB2-a* is a PKA-dependent activator (Fig. 6). Transfection of expression constructs for PKA or *dCREB2-a* alone gave only modest activation of the CRE

reporter above baseline values. Cotransfection of these constructs together, however, gave levels of activation 5.4-fold greater than the activation seen with PKA alone. This level of PKA-dependent activation was only slightly less than that obtained with mammalian CREB-341 in parallel experiments (data not shown).

dCREB2-b did not function as a PKA-dependent activator in this assay. It failed to stimulate CRE reporter activity in the presence or absence of PKA (data not shown). Instead, *dCREB2-b* worked as a direct antagonist of PKA-dependent activation by *dCREB2-a* (Fig. 7). Cotransfection of equimolar amounts of the *dCREB2-a* and *dCREB2-b* expression constructs, along with a construct expressing PKA, resulted in a

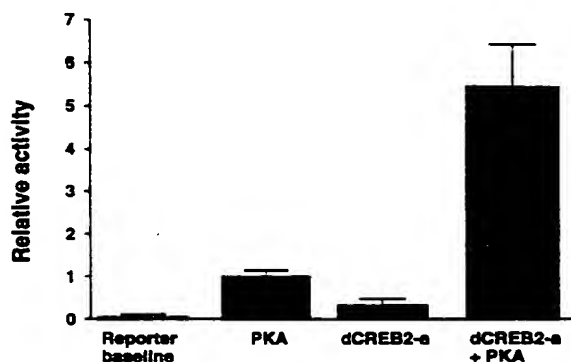


FIG. 6. PKA-responsive transcriptional activation by *dCREB2-a*. F9 cells were transiently transfected with 10 μ g of $\Delta(-71)$ CAT plasmid as a CRE-directed reporter; 5 μ g of RSV- β gal reporter was included in each dish as a normalization control for transfection efficiency. Different groups received 8 μ g of *dCREB2-a* expression vector and 4 μ g of PKA expression vector, separately or in combination, as indicated. All results are expressed as CAT/ β -galactosidase enzyme activity ratios, standardized to values obtained with PKA-transfected dishes.

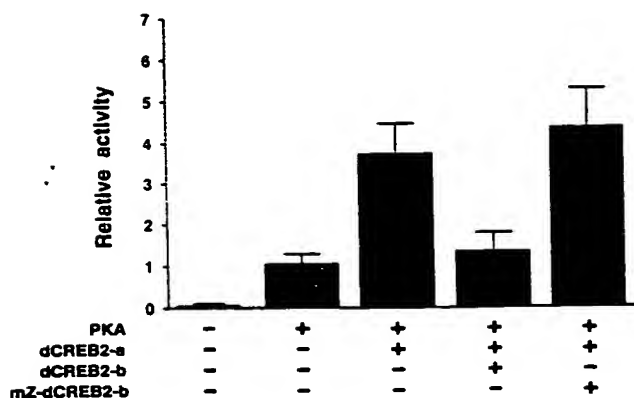


FIG. 7. Transcriptional effect of dCREB2-b and a mutant variant on PKA-responsive activation by dCREB2-a. F9 cells were transiently cotransfected with 10 μ g of $\Delta(-71)$ CAT along with the indicated combinations of the following expression constructs: RSV-dCREB2-a (5 μ g), pMtC (2 μ g), RSV-dCREB2-b (5 μ g), and RSV-mLZ-dCREB2-b, which expresses a leucine zipper mutant of dCREB2-b (5 μ g). The DNA mass for each dish was made up to 27 μ g with RSV-0. Other experimental conditions are as described in the legend to Fig. 6.

nearly complete block of the PKA-dependent activation produced by dCREB2-a.

Near identity between the predicted leucine zippers of dCREB2 and mammalian CREB suggested that the effects of mutations in CREB in this region could serve as a guide for making mutations in dCREB2. A DNA coding for a mutant dCREB2 molecule, mLZ-dCREB2-b, was made by introducing two single-base changes that convert the middle two leucines of the predicted leucine zipper to valines. An identical mutation in CREB abolishes homodimerization in vitro (17). In cotransfection experiments, expression of a construct for mLZ-dCREB2-b failed to block PKA-dependent activation by dCREB2-a (Fig. 7).

DISCUSSION

We have investigated the dCREB2 gene and found that it encodes the first known PKA-responsive CREB transcriptional activator in *D. melanogaster*. Previously, the mammalian CREB, CREM, and ATF-1 genes were the only CREB family members known to express PKA-responsive transcriptional activators. These three mammalian genes form a CREB subfamily defined by this shared function and by amino acid homology, which is especially strong in the bZIP region. A protein database homology search indicated that dCREB2-a is most similar to CREB, CREM, and ATF-1 proteins, especially in the bZIP region, where up to 90% amino acid identity was found. For these reasons, we propose that dCREB2 is a member of this subgroup of CREB family genes that produce PKA-responsive transcriptional activators and thus might play roles in *D. melanogaster* analogous to those served by the mammalian genes in this group.

The dCREB2 transcript undergoes alternative splicing. dCREB2-a, -b, -c, and -d are splice forms that predict variants of the activation domain attached to a common bZIP region. These alternative splice forms result in seemingly minor changes in the size and spacing of parts of the activation domain. Nevertheless, alternative splicing of the activation domain has profound effects on the functional properties of dCREB2 products. dCREB2-a is a PKA-responsive transcriptional activator in cell culture, whereas dCREB2-b, lacking

exons 2 and 6, produces a specific antagonist. Preliminary data (not shown) suggest that dCREB2-c and dCREB2-d are not activators, suggesting that the activator form requires both exons 2 and 6. Although we have not detected a cDNA which corresponds to dCREB2-a (which is a composite splice form inferred from our cDNA and RT-PCR analysis), we have shown that this molecule functions both in cell culture (Fig. 6) and in transgenic flies (76) as a PKA-responsive activator. This dCREB2 splicing pattern (and its functional consequences) is virtually identical to that seen in the CREM gene. In CREM, alternative splicing of exons flanking the P box determines whether a particular isoform is an activator or an antagonist (23, 25, 42). Activator forms contain the alternatively spliced exons, while blocking forms are missing the exons.

In contrast to the dCREB2 splicing variants that encode isoforms with a bZIP domain, the dCREB2-q, -r, and -s splice forms incorporate in-frame stop codons, resulting in predicted proteins which are truncated amino terminal to the bZIP region. Isoforms of this type have been identified among the products of the CREB gene (13, 64) but not among those of the CREM gene. The function of these truncated molecules is not known, but at least one such CREB mRNA is cyclically regulated in rat spermatogenesis (75).

Other CREBs have been identified in *D. melanogaster*, but dCREB2-a is, so far, the only cAMP-responsive *Drosophila* CREB transcription factor. These other *Drosophila* proteins, BBF-2/dCREB-A (1, 67) and dCREB1 (76a), have substantially less amino acid homology to mammalian CREB, CREM, and ATF-1 than does dCREB2. It may be that in *D. melanogaster*, dCREB2 is the only representative of this family of genes.

Protein homology and structural gene similarity between the mammalian CREB and CREM genes has led some to suggest that these genes may be the result of a gene duplication (48, 64). The *Drosophila* dCREB2 gene has mRNA splicing isoforms similar to exclusive products of CREB and CREM. In combination with amino acid sequence homology and the functional similarity between the predicted proteins from dCREB2 and those from CREB, CREM, and ATF-1, the variety of dCREB2 splice products suggests that dCREB2 may be an ancestral form of the mammalian family of PKA-responsive CREB genes.

We believe that the net level of gene activation by CREB family members in response to activation of the cAMP pathway depends on the ratio of the amounts and activities of activator isoforms to blocking isoforms, a proportion that we call the A/B ratio (76). In mammals, three genes (CREB, CREM, and ATF-1) can possibly contribute to this ratio, thus making it difficult to evaluate the function of any single gene in a particular process. For instance, CREB knockout mice appear normal, but at the molecular level show increased levels of CREM expression (37), showing that compensation can occur among genes of this group. In other experiments, expression of a CREB blocker transgene under control of the somatostatin promoter in mice results in dwarfism (70), while the knockout mice are normal in size.

If dCREB2 is the only PKA-responsive CREB in *D. melanogaster*, experiments aimed at disrupting CREB-mediated cAMP-responsive transcription may be easier to perform and interpret in this organism than in the multigene mammalian systems. Although *D. melanogaster* apparently uses only a single gene, the dCREB2 gene retains many of the subtleties of its mammalian counterpart, including the production of both activators and blockers. By combining the well-developed molecular genetic techniques available for *D. melanogaster* with the ability to make inducible transgenic flies and isolate mutations,

dCREB2 could provide insights not currently available elsewhere into the role of cAMP-responsive transcription in a variety of biological processes.

One such process is the possible involvement of CREB in long-term memory formation. Results from studies with *Aplysia californica* pointed to a requirement for a CREB factor in a cellular model for long-term memory (2, 12). Recently we have performed experiments with *D. melanogaster* to test the effects of blocker or activator *dCREB2* isoforms on long-term memory in the intact, behaving animal. Using a conditionally expressed transgene, we have shown that induced *dCREB2-b* expression specifically abolishes long-term memory (77). Most recently, we have obtained a complementary result in *dCREB2-a* transgenic flies, in which induction of the activator actually potentiates formation of long-term memory (76). We believe that long-term memory depends on PKA-responsive gene activation via CREBs and that this transcriptional switch is a function of the A/B ratio in relevant brain cells during and after behavioral training.

Discovery of a cAMP-responsive transcriptional control system in *D. melanogaster* can also be rapidly integrated into the framework of ongoing molecular genetic investigations. Two examples of where this is occurring are biological rhythms and development. Rhythmic phosphorylation of CREB and cyclic expression of a blocking CREM isoform, ICER, is correlated with a phase of a biological clock in mammals (26, 69, 72). Mutations directly affecting cAMP metabolism are reported to alter behavioral rhythms in *D. melanogaster* (44), in which a clock gene has been cloned (20, 78). Experiments evaluating the effects of inducing *dCREB2* transgenes on behavior or expression of the clock protein may prove informative.

Lane and Kalderon (41) showed the general involvement of the cAMP second-messenger system in *Drosophila* development by using mutants in the catalytic subunit of PKA. Recently, the *hedgehog* developmental pathway has become the subject of intense interest in both *Drosophila* and mammalian systems (6, 19, 35, 53, 62), and results of studies with *D. melanogaster* suggest a role for cAMP in this system (10a, 37a, 63a, 69a). The possible involvement of CREB-mediated transcription in these processes now can be tested by using reverse genetic strategies.

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